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Topical vitamin C protects porcine skin from ultraviolet radiation-induced damage

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Summary Ultraviolet radiation damage to the skin is due, in part, to the generation of reactive oxygen species. Vitamin C (L-ascorbic acid) functions as a biological co-factor and antioxidant due to its reducing properties. Topical application of vitamin C has been shown to elevate significantly cutaneous levels of this vitamin in pigs, and this correlates with protection of the skin from UVB damage as measured by erythema and sunburn cell formation. This protection is biological and due to the reducing properties of the molecule. Further, we provide evidence that the vitamin C levels of the skin can be severely depleted after UV irradiation, which would lower this organ's innate protective mechanism as well as leaving it at risk of impaired healing after photoinduced damage. In addition, vitamin C protects porcine skin from UVA-mediated phototoxic reactions (PUVA) and therefore shows promise as a broad-spectrum photoprotectant.

In a spate of research activity over the past few years, the conclusion that oxygen free radicals are involved in cutaneous sun damage has become well accepted.[1–4] The proposal that the skin's native antioxidant protection system breaks down under this 'photo-onslaught' has been suggested; for example, it has been shown that in response to acute UV exposure, epidermal superoxide dismutase activity declines.[5–6] Glutathione reductase, catalase and reduced glutathione levels in skin have also been shown to decline after only one exposure to UVB radiation;[7] except for glutathione, the same results hold for UVA-visible irradiation of skin.[8] Loss of other skin antioxidants (ubiquiones, vitamins E and C, etc.) ranged from minimal to significant. Prophylactic antioxidant therapy has been investigated in so far as it has been shown that increased dietary antioxidants (including vitamin C) lessen UV-induced skin lesions,[9,10] that topically applied, liposomally encapsulated superoxide dismutase reduces a UV-mediated loss of the native enzyme[11] and that free-radical scavengers inhibit ultraviolet radiation's formation of 'sunburn cells' in excised skin.[12] In addition, topical application of several free-radical scavengers on guinea-pig skin lessened subsequent photosensitized reactions (erythema and oedema).[13] Finally, a recent report has shown topically applied vitamin E or vitamin C effective in moderating low level, chronic UVB (but not UVA) damage to mouse skin.[14]

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Vitamin C (L-ascorbic acid) has received renewed attention as an antioxidant of biological importance in systems varying from blood ('. . . an outstanding antioxidant in human blood plasma'[15]) to skin.[16] On the skin surface, it and thioredoxin reductase may be very important in eliminating reasonably long-lived free radicals.[16,17] Although vitamin C is valuable as a free-radical quencher, it may itself be susceptible to UV-mediated destruction. As such, we investigated the ability of pharmacological amounts of topically applied vitamin C to increase cutaneous concentration of this vitamin and to lessen UV damage to porcine skin.

Methods

L-Ascorbic acid (A.C.S. reagent), D-isoascorbic acid, 1,2 propanediol (propylene glycol) and hydroxypropylcellulose (average MW = 300,000) were purchased from Aldrich. Experiments utilized a 10% L-ascorbic acid (w/v) solution in 20% propylene glycol (v/v) with 0·5% hydroxypropylcellulose (w/v) incorporated as thickener. Stored at 4°C, this solution was stable for 1 week as measured spectrophotometrically.

Animals

Domestic Yorkshire pigs (males weighing 32–45 kg) were used in this study because of the similarity of their skin to humans. They were housed in barns at the North Carolina State Veterinary School and fed a standard diet. Prior to experiments, animals were anaesthetized with ketamine/xylazine (2 mg/kg) and their backs clipped with animal shears. During irradiation or before biopsy, all animals were additionally placed under halothane anaesthesia. Typically four animals were used per experiment.

UV source

A bank of two Westinghouse FS40 lamps was used in the bulk of the studies. They were placed above a restrained animal at a distance of approximately 10 cm. The intensity at that distance measured 0·45–0·5 mW/cm^2 using a National Biological Corporation model UVB LMH06C photodetector. Alternatively, specially made Philips TL 40W/01 fluorescent bulbs (four housed in a planar array) were used experimentally. They were placed above the restrained animals to achieve an intensity of $\approx$0·8 mW/cm^2. In the PUVA experiments, four GE F40 BL fluorescent bulbs were used. These were placed above the animals so that the measured intensity was 2 mW/cm^2 (using an International Light Model IL 440 detector). In these experiments, 8-methoxypsoralen was formulated at a 0·1% (w/v) concentration in 90% ethanol. The solution was applied to the sites at 10 μg/cm^2. Additional experimental details are given in the Figure and Table legends.

Percutaneous absorption

Skin was removed from a non-treated animal at the time of killing to a depth of 500 μm with a Padgett Model B dermatome. The skin was cut into 1·5-cm squares and placed between the upper and lower chambers of a modified Franz cell. Vitamin C samples (20 μl) containing 1 μCi ^{14}C-L-ascorbic acid (Amersham) were pipetted on to skin in multiple cells. At 24 and 48 h, 100-μl aliquots were removed from the buffered saline reservoir, mixed with scintillant and counted on an LKB model 1219 scintillation counter. The skin sample itself was then washed by dipping for 10 s into three consecutive water baths (this was shown to remove virtually all surface counts) and burned in a stream of oxygen in a Packard model 306 tissue oxidizer. The $^{14}CO_2$ released was trapped in scintillant and also counted. Combination of the the two values gave an estimate of the penetration of the ascorbic acid into and through the epidermis.

Histology

The experimental sites were biopsied with a 4-mm punch at 24 h for UVB experiments. For UVA experiments, biopsies were taken at 48 h. Like humans,[18] these Yorkshire pigs showed variable, delayed responses to PUVA treatment. The biopsies were fixed in formalin and processed for routine histology. For 'sunburn cell' analysis, duplicate haematoxylin and eosin-stained sections of triplicate biopsies for each experimental site were analysed for sunburn cells (basal keratinocytes having pyknotic nuclei as well as eosinophilic cytoplasm) and normalized to the 4-mm punch diameter. The number of sunburn cells per given condition was calculated in this way from four animals per experiment.

HPLC analysis of vitamin C levels in skin

To determine vitamin C concentrations in the skin, HPLC with electrochemical detection was used.[19] Briefly, after extensive washing, skin was excised at the subcutaneous boundary from two or more animals using a 4-mm punch, and immediately frozen in liquid nitrogen. Weighed skin samples were placed on a glass dish in cold 5% metaphosphoric acid (MPA) and finely minced with surgical scalpels. Following a wash with additional MPA the minced skin sample was subjected to three cycles of freeze-thawing (-70°C). Samples were spun down and the supernatant filtered through a 0·22 μm filter. All samples were frozen at -70°C until analysis.

Laser-Doppler measurement of cutaneous blood flow

Laser-Doppler velocimetry (LDV) is an accepted non-invasive technique for monitoring relative blood flow and has been used in quantifying erythema caused by UV irradiation.[20] Cutaneous blood flow was estimated by LDV using a LaserFlo™ BPM model 403 24 h after UV irradiation of vitamin C or vehicle-treated sites on four animals.

Results

Skin levels of vitamin C after topical treatment with this vitamin

Using modified Franz cells, percutaneous absorption studies with ^{14}C-labelled ascorbic acid showed that

Table 1. Increase in cutaneous vitamin C levels by topical treatment with this vitamin. Four-millimetre biopsies from each treatment site on each of three animals were weighed, pooled and extracted as in Methods. Samples were analysed in duplicate by HPLC with electrochemical detection and peak areas compared to known standards. Values are mean ± SE of duplicate analyses of two pooled samples.

Site	Vitamin C concentration μg/g wet weight
Vehicle-treated	20·2 ± 1·9
Vitamin C-treated	542·6 ± 50·1*

* $P < 0{\cdot}005$, Student's *t*-test.

0·7 ± 0·1% (mean ± SEM) passed through and 8·2 ± 1·1% (mean ± SEM) of the applied dose (2 mg) was present within a 500-μm thick section of porcine skin 48 h after topical application. However, this technique only follows the movement of the radioactive label and as vitamin C is notoriously unstable, we also measured cutaneous levels of vitamin C proper by HPLC. Table 1 is derived from an experiment in which three animals were treated topically with vitamin C, then thoroughly washed, and full thickness skin samples excised and assayed for ascorbic acid levels. Additional experiments with different animals have confirmed that skin levels of vitamin C increase 4–40-fold or more following multiple treatments with this vitamin. Assuming porcine skin to be approximately 75% water (a fair approximation), both methods give values of vitamin C concentration in the skin of approximately 3–4 mM after topical application. Because of the obvious gradient, even higher levels of this vitamin can be assumed in the upper levels of the epidermis and stratum corneum while somewhat lower in the reticular dermis.

Protection of swine skin from UVB by topical vitamin C

Topical application of vitamin C protects porcine skin from UVB damage. Using sunburn cell (dyskeratotic basal epidermal cells) formation as a marker for UV-induced damage, Table 2 shows a significant reduction in the UV-mediated formation of this cell type by topical application of vitamin C. Although a single application (approximately 15–30 min prior to exposure) is generally sufficient to give UV protection, some animals showed better results if the vitamin C was applied more than once, and as such, a typical protocol called for several applications of the vitamin to the skin prior to irradiation.

The protection noted in Table 2 is not, however, due to a 'sunscreen effect'. The UV source in those experiments

Table 2. Reduction in UVB-mediated sunburn cell formation by topical vitamin C. Paired sites on the backs of domestic pigs were pretreated for 3 days, and 30 min before irradiation, with a 10% vitamin C solution or the vehicle control (100 μl/10 cm^2). The sites were then irradiated with the indicated dose of UVB from a bank of Philips TL 40W/01 bulbs. At 24 h, three biopsies were taken from each site. Duplicate sections from each biopsy were stained with haematoxylin and eosin and the number of 'sunburn cells' counted. Cell counts from the six histological sections per condition were normalized to the 4-mm biopsy diameter. Values are the mean ± SE. Numbers in parentheses equal animal number. From this UV source in these animals 100 mJ/cm^2 ≈ 1 MED

Condition	No. of sunburn cells/4-mm biopsy (dose 400 mJ/cm^2)
Control (12)	33·1 ± 5·1
Vitamin C-treated (12)	20·5 ± 3·0*

* $P < 0{\cdot}005$; paired *t*-test.

was a bank of four Philips TL 40W/01 bulbs whose emission centres around 311 nm with little or no output below 305 nm (Fig. 1). The L-ascorbic acid in this formulation has virtually no absorbance above 300 nm. Indeed, if this solution is applied to a piece of quartz glass in a similar manner to that employed experimentally (≈ 100 μl/10 cm^2) and allowed to air dry for 30–60 min, virtually 100% of the energy at wavelengths greater than 300 nm is transmitted through the quartz cuvette. Therefore, the protection seen against UVB is biological, not physical. Responses to these Philips bulbs (sunburn cell formation and erythema) were indistinguishable from those seen with more typical UVB bulbs, e.g. Westinghouse FS40.

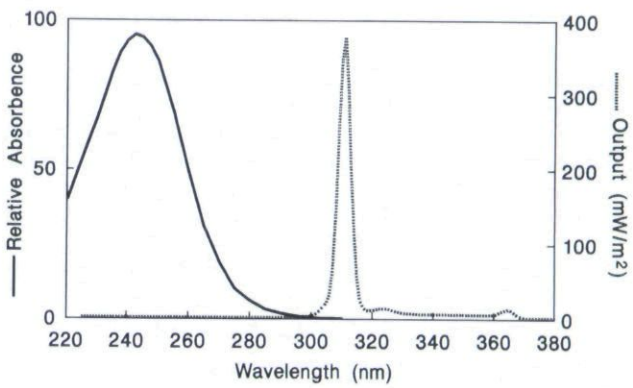

Figure 1. Absorption spectrum of L-ascorbic acid versus emission spectrum of Philips TL 40W/01 fluorescent bulbs. The absorbence of the vitamin C in the test solution was measured through quartz cuvettes using a Shimadzu Model 260 spectrophotometer. The emission spectral data was obtained from the manufacturer. (—) Relative absorbence of L-ascorbic acid in experimental formulation. (- - - -) Emission spectrum of TL 40W/01 fluorescent bulb.

Table 3. Reduction in UVB-elicited erythema by topical vitamin C. Paired sites on the backs of domestic pigs were pretreated for 1 week, and 30 min before irradiation, with a 10% vitamin C solution or the vehicle control. The sites were then irradiated with approximately 2–3 MED of UVB radiation (≈ 150 mJ/cm^2 from two Westinghouse FS40 bulbs). At 24 h, triplicate measures of cutaneous blood flow from each site were taken using a TSI LaserFlo™ model 403 laser-Doppler velocimeter. Adjacent non-irradiated skin was used for 'background' blood flow determination. Values are mean ± SE of arbitrary blood flow values after subtraction of 'background' flow. Numbers in parentheses equal animal number

Condition	Cutaneous blood flow
Control (4)	2·91 ± 0·50
Vitamin C-treated (4)	1·40 ± 0·70*

* $P < 0{\cdot}001$; paired *t*-test.

A second hallmark of UVB-induced sunburn is erythema. One quantitative measure of erythema is provided by laser-Doppler velocimetry. Using this technique, increased skin blood flow occurred 24 h after a 2-3 MED dose of UVB was measured. In this experiment, blood flow approximately doubled compared with adjacent non-irradiated control sites in response to the radiation. Pretreatment with topical vitamin C halved this increase (Table 3).

Protection against UVB damage by reducing and non-reducing analogues of vitamin C

The antioxidant protection of skin by vitamin C should be mimicked by its isomer isoascorbic acid, assuming cellular uptake and/or *in vivo* penetration rates are similar for the two molecules (not an absolute, e.g. Kipp and Schwartz[21]). Table 4 shows that both ascorbic acid and isoascorbic acid exhibit identical protective abilities against UVB damage. In a separate experiment, the two-electron oxidation product of ascorbic acid, dehydroascorbate (DHA), provided little or no visual protection against UV erythema when compared with its parent molecule (data not shown).

Table 4. Comparative effectiveness of ascorbic acid vs. isoascorbic acid in preventing UVB-induced 'sunburn cell' formation. Solutions were 10% (w/v) in 20% propylene glycol; 1·0% hydroxy-propylcellulose. Paired sites on the backs of domestic pigs were treated daily for 1 week, and 30 min before irradiation, with 2–3 MED of UVB (Westinghouse FS40 bulbs). After 24 h, 4 mm biopsies (three per site) were taken, processed for histology, stained with haematoxylin and eosin and 'sunburn cells' counted as in Table 2. Numbers in parentheses equal animal number.

Treatment	No. of sunburn cells/4 mm	% Reduction
Vehicle (8)	17·2 ± 6·0	
L-ascorbic acid (8)	10·0 ± 3·7*	42
Vehicle (8)	18·3 ± 4·5	
isoascorbic acid (8)	10·8 ± 2·5*	41

* $P < 0{\cdot}02$; paired *t*-test.

Protection of pig skin from UVA-mediated phototoxicity by topical vitamin C

Although nowhere near as erythemogenic or carcinogenic as UVB, UVA is responsible for many biological effects, at least partially because the relative intensity of UVA in solar UV is many times that of UVB. Because of the aforementioned ineffectiveness of low-dose UVA to elicit measurable biological responses in skin, we chose to use 8-methoxypsoralen plus UVA (PUVA) to increase skin damage, i.e. sunburn cell formation, and allow us to quantify possible protection by topical vitamin C. This method has been used to test UVA sunscreen efficacy, and to assess the role of reactive oxygen in PUVA damage *in vivo*.[13,22–24] Table 5 shows that topical application of vitamin C does inhibit the PUVA-mediated production of this cell type, indicating that vitamin C is capable of ameliorating a UVA-mediated phototoxic response in skin. Indeed, in experiments using UVA doses greater than 500 mJ/cm^2, gross pathological changes (leucocytic infiltrates, blistering, etc.) are seen

Table 5. Reduction in psoralen-UVA-mediated sunburn cell formation by topical vitamin C. Paired sites on the backs of domestic pigs were pretreated for 3 days with a 10% vitamin C solution or the vehicle control. One hour prior to irradiation, 8-methoxypsoralen (0·1% w/v in 90% ethanol) was evenly applied to all sites (10 μg/cm^2). Thirty minutes later a final treatment of vitamin C or vehicle (100 μl) was applied. The sites were then irradiated with a bank of GE F40 BL fluorescent tubes (emission peak ≈ 360 nm) at the indicated dose. In these animals, this is approximately equal to three times the minimum phototoxic dose (MPD). Biopsies were taken at 48 h. Sunburn cell analysis was performed as in Table 2. Numbers in parentheses equal animal number

Condition	No. of sunburn cells/4-mm biopsy (dose 500 mJ/cm^2)
Control (10)	114·5 ± 17·8
Vitamin C-treated (10)	48·4 ± 10·1*

* $P < 0{\cdot}002$; Paired *t*-test.

Table 6. UV-mediated loss of cutaneous vitamin C. Sites on three animals were treated with topical vitamin C (daily for 5 days). One site was then covered and the other exposed to approximately 4 MED of UVB. Immediately after the exposure, the sites were thoroughly washed. Multiple biopsies were taken from the two sites on each animal, frozen in liquid nitrogen and stored at −70°C until analysed. In two separate analyses, biopsies from the irradiated or non-irradiated sites from the three animals were pooled, weighed and extracted for HPLC quantification of vitamin C content. Vitamin C-treated, non-irradiated values were treated as 100% and irradiated values compared with them. Numbers represent the average of five HPLC determinations per experimental condition.

Treatment	% Vitamin C remaining
Vitamin C-treated	
Non-irradiated	100
Irradiated	34

in vehicle-treated sites, whereas vitamin C pretreated sites maintained substantially normal histology (unpublished data). No evidence was found for any interaction between vitamin C and the ground-state psoralen molecule (measured spectrophotometrically) so the protection is presumed to be due to either quenching of the excited state psoralen molecule by vitamin C or its scavenging of reactive oxygen species known to be generated during PUVA therapy.[13]

Loss of cutaneous vitamin C by UV radiation

Vitamin C is essential to tissue repair, a fact noted many years ago. We, and others,[5–7] hypothesized that UV damage may in part be due to a depletion of the skin's natural defences by the radiation. In fact, Fuchs *et al.*[7] have shown UV-mediated losses of several key antioxidants in mice exposed to UVB. Vitamin C levels (measured spectrophotometrically) declined, although not significantly. We found it difficult to document ultraviolet radiation-induced changes in vitamin C concentration in full thickness skin; vasodilatation and skin inflammation may lead to variable levels. To circumvent this problem, we used several days of topical vitamin C treatment to raise skin levels of the vitamin. The treated areas were then UVB-irradiated, washed thoroughly to remove any free vitamin C, and the cutaneous vitamin C concentration measured by HPLC. Table 6 shows a dramatic UV-mediated diminution of the tissue vitamin C. It thus appears likely that the natural vitamin C levels of the skin are also subjected to this loss, perhaps to near depletion in areas.

Discussion

Although the mechanism(s) of ultraviolet damage to skin is certain to be complex, reactive oxygen species are likely to be a major contributor to this damage. Unlike model systems, UV probably generates the entire spectrum of oxygen radicals, radical-derived species and other reactive molecules. In this regard, ascorbic acid should be particularly effective in interfering with the UV-mediated generation and/or propagation of the reactive oxygen species, as it reacts with or quenches the superoxide anion,[25] the hydroxyl radical,[26] singlet oxygen[27] and hypochlorous acid.[28] In addition, vitamin C is the primary replenisher of vitamin E, the preeminent inhibitor of lipid peroxidation.[29,30] Durham *et al.* showed that increases in dietary vitamin C led to a statistically significant reduction in subsequent UV-elicited tumours.[10] We believed that topical application would achieve a much higher tissue concentration of this vitamin compared with oral intake. Experiments are in progress to specifically test this. However, conservative calculations based on the percutaneous absorption and HPLC experiments give ascorbic acid concentrations in the millimolar range in full or partial thickness skin after several days of topical application. These levels would be expected to be even higher in the superficial layers of the skin, i.e. the epidermis and upper papillary dermis. *In vitro* evidence suggests that at these concentrations ascorbic acid strictly acts as an antioxidant.[31] Experiments with the isoascorbate and dehydroascorbate also show the effects to be biological and dependent on the reducing property of this vitamin. It is of note that, although it is known that dehydroascorbate can be metabolically converted back to the parent ascorbic acid molecule within cells, this apparently does not occur under our experimental conditions, or more likely, the bulk of the protection occurs in the metabolically inactive cell layers, i.e. the stratum corneum. In defense of this thesis, using vitamin C solutions at either pH 2·5 (totally un-ionized) or 5·0 (totally ionized) makes no difference in protection against UVB damage even though the un-ionized form traverses the skin to a much greater degree (data not shown). In addition, as previously mentioned, protection is noted after only one application of the vitamin, 15–30 min before UV exposure, presumably long before dehydroascorbate could reach the metabolically active layers of the epidermis. Thus, it is not unexpected for DHA to be inactive in this model and reasserts the importance of the reductant nature of vitamin C in the lessening of damage noted.

These studies in part employed fluorescent UVB-emitting bulbs, wavelengths long known to be responsible for eliciting skin cancer. Multiple exposures to significantly lower doses than used in this study has been shown to be carcinogenic.[32] Additionally, reactive oxygen species/oxygen radicals have been implicated in the tumour promotion phase.[32,33] One study has shown a positive effect of topical vitamin C in preventing the onset of tumourogenesis in mice.[14] This is an ongoing avenue of study.

Quantitative measures (sunburn cell counts and laser-Doppler measurements) show that topical vitamin C treatment can reduce UVB-mediated increases in these parameters $\approx 40\%$ at moderate UV doses (3–4 MED). Tissue hypoxia has also been shown to reduce UVB-induced formation of suburn cells, again by approximately 40–50%.[34] These data suggest that, although important, reactive oxygen species cannot be considered the only source of UVB damage. Certainly, historical experiments showing direct (non-oxygen-dependent) interaction between UV (particularly UVB) and important biological targets, e.g. DNA, are well known. Vitamin C obviously need not interfere with these other reactions.

Recent studies hint at a potential drawback to many ordinary sunscreens. They absorb the sunburning rays of the sun so effectively that they allow the user to stay outdoors much longer than would ordinarily be possible. Unfortunately, this allows considerable amounts of the longer wavelength, non-absorbed UVA to enter the skin, which may be related to an increased risk of certain skin cancers or ageing changes. In addition, there appears to be a stronger correlation between UVA-induced skin damage and oxygen radical formation than with UVB.[35] As we have now shown, topical vitamin C is also effective in moderating UVA-mediated phototoxic reactions in skin, apparently due to its antioxidant status. Earlier *in vitro* work showed that ascorbic acid and anaerobiosis were equally effective in inhibiting PUVA-mediated intermolecular cross-link formation of α-crystallin, providing further evidence for the participation of reactive oxygen species in at least some psoralen-UVA photosensitized reactions.[36] These results are in contrast with investigations performed in mice, i.e. vitamin C was ineffective in preventing UVA-induced skin wrinkling.[14] Considerable differences, e.g. long-term UVA exposure vs. acute PUVA treatment, mouse vs. pig skin, as well as measured endpoints, exist between these two studies. Further studies will be necessary to understand the reasons for these differences. Whether the mechanisms responsible for PUVA damage in skin are the same as those leading to chronic UVA damage is a subject for debate. As there are no good models for UVA damage alone, PUVA has become an acceptable experimental model for predicting product efficacy in providing protection against longer wavelength ultraviolet radiation (UVA).[23,24] Topical vitamin C treatment may thus prove to be a particularly effective broad spectrum photoprotectant.

Participation of reactive oxygen species in, and antioxidant prevention of, UV damage is now well documented.[1–4,13,14,37] We report that topically applied L-ascorbic acid is effective in preventing UVB- and psoralen-UVA-mediated damage to pig skin. The mechanism(s) of this protection is presumed to be due to its 'antioxidant' status. The protection may be due to vitamin C directly reacting with, or quenching certain reactive species, or by regenerating vitamin E and thus participating in the inhibition of UV-induced lipid peroxidation. As significant free-radical reducing potential is found in the extracellular spaces,[16] topical vitamin C may significantly bolster this free-radical reducing capacity and thus benefit the skin. The skin's innate antioxidant defense system is complex. We believe that vitamin C is an important part of the UV defense system of the skin, although its exact role has not yet been fully elucidated.

Finally, vitamin C is essential to tissue and/or wound repair. Perhaps UV damages skin not only directly, but indirectly via inactivation of protective agents such as superoxide dismutase, catalase, etc. We provide evidence that additionally, skin vitamin C levels can be dramatically reduced in response to UV radiation. Thus, in addition to a loss of antioxidant status, it may be hypothesized that other tissue damage may be less effectively repaired in this 'vitamin deficient' state. Therefore, replenishment of skin vitamin C would be an important pharmacological intervention against sun damage.

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